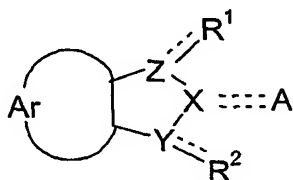

Inhibitors of DNA methylation in tumor cells

5 The present invention relates to compounds of the general formula (I), with the definitions of R^1 to R^2 given below in the text, and the use of these compounds and/or pharmaceutically acceptable salts thereof as a pharmaceutical. The compounds according to formula (I) lend themselves in particular as inhibitors of DNA methylation in cells,
10 particularly tumor cells.



(I)

15 DNA can be methylated through covalent methylation of cytosine residues at their carbon-5 position. It has been found that DNA methylation is an important mechanism of gene regulation, particularly gene silencing. Gene regulation by DNA methylation is an "epigenetic" form of gene regulation, as the DNA sequence information itself remains
20 unaltered.

Aberrant DNA methylation patterns are closely associated with epigenetic mutations or epimutations, which can have the same consequences as genetic mutations. For example, many tumors show hypermethylation and concomitant silencing of tumor suppressor
25 genes. Several developmental disorders are also associated with aberrant DNA methylation.

Thus, changes in DNA methylation play an important role in developmental and proliferative diseases, particularly in tumorigenesis.

30 The DNA methylation reaction is catalyzed by DNA methyl transferases (DNMTs). Establishment and maintenance of DNA methylation patterns require the activity of several DNMTs. In mammals, DNA methylation is established during early embryogenesis by

the de novo DNA methyl transferases (DNMT3A and DNMT3B). In differentiated cells, methylation patterns are maintained by DNMT1 which is therefore also responsible for maintenance of epimutations.

- 5 Inhibition of DNA methylation, particularly by inhibition of DNMTs, more particularly DNMT1, is considered a promising strategy for treatment of proliferative diseases.

However, satisfying methods for inhibition of DNA methylation have previously not been available.

10

Genetic inhibition of DNMTs, although theoretically possible (US 6,054,439), suffers from the widely known and currently insurmountable difficulties associated with gene therapy.

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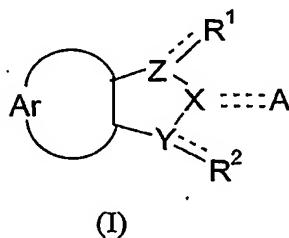
Pharmacological inhibition of DNMTs has been limited to the use of structural analogues of cytosine, such as 5-azacytidine, 5-aza 2'deoxyctidine (decitabine), and 5,6-dihydro-5-azacytidine (US 4,058,602; DE 198 23 484 A1). These cytosine analogues suffer from low specificity and high toxicity, limiting their use to a very small set of clinical indications.

20

Therefore, there is a need for other inhibitors of DNA methylation, particularly inhibitors of DNMTs. Preferably, such inhibitors should have a different mode of action than structural analogues of cytidine, and they should be more specific and less toxic than other inhibitors of DNA methylation. The object of the present invention is to provide compounds and pharmaceuticals showing said abilities.

25

This object is attained by compounds according to the general formula



30

wherein the dotted lines denote a single bond which is optionally present, with 1 dotted line and 1 full line or 2 dotted lines denoting a double bond; wherein, in case no double bond is present and a free valence exists, this valence is occupied by H; and wherein the symbols have the following meanings:

R^1 and R^2 are independently from each other selected from the group consisting of:
H; (=O); OH; OSO_3^- ; halogens; pseudohalogens; NR^3R^4 ; $\text{S(O)}_m\text{R}^5$; $\text{SO}_2\text{NR}^6\text{R}^7$; $\text{C(O)}\text{R}^8$;
5 $\text{C(O)}\text{OR}^9$; $\text{CONR}^{10}\text{R}^{11}$; substituted and unsubstituted $\text{C}_1\text{-C}_3$ -alkyl and substituted and
unsubstituted $\text{C}_1\text{-C}_3$ -alkoxy, which alkyl and alkoxy groups, if substituted, carry at least
one substituent from the group: OH, OSO_3^- , halogens, pseudohalogens, NR^3R^4 , $\text{S(O)}_m\text{R}^5$,
10 $\text{SO}_2\text{NR}^6\text{R}^7$, $\text{C(O)}\text{R}^8$, $\text{C(O)}\text{OR}^9$, $\text{CONR}^{10}\text{R}^{11}$, and wherein at least one substituent is
different from H;

Ar denotes a substituted or unsubstituted mononuclear aryl group having 6 or 7 members,
10 which aryl group is annulated to the neighbouring 5-membered cycle, and which may carry
1, 2 or 3 heteroatoms from the group N, O and S in its cycle;

Y, Z denote independently from each other a nitrogen atom, an oxygen atom, a sulfur atom
or a methylene group;

15 X is a nitrogen atom, an oxygen atom, a sulfur atom, or a methylene group;

A is selected from the group consisting of: H; halogens and pseudohalogens; OH; $=\text{N(OH)}$;
 $\text{NR}^{12}\text{R}^{13}$; OSO_3^- ; $\text{S(O)}_m\text{R}^{14}$; $\text{SO}_2\text{NR}^{15}\text{R}^{16}$; $\text{C(O)}\text{R}^{17}$; $\text{C(O)}\text{OR}^{18}$; $\text{CONR}^{19}\text{R}^{20}$; $\text{C(S)}\text{R}^{21}$,
20 $\text{C(S)}\text{OR}^{22}$; unsubstituted and at least monosubstituted $\text{C}_1\text{-C}_{12}$ -alkyl which can carry in its
chain one or more non-adjacent heteroatoms from the group nitrogen and oxygen, and
which, if substituted, carry at least one substituent which is preferably selected from the
group consisting of: halogens, pseudohalogens, OH, $\text{NR}^{12}\text{R}^{13}$, OSO_3^- , $\text{S(O)}_m\text{R}^{14}$,
 $\text{SO}_2\text{NR}^{15}\text{R}^{16}$, $\text{C(O)}\text{R}^{17}$, $\text{C(O)}\text{OR}^{18}$, $\text{CONR}^{19}\text{R}^{20}$, $\text{C(S)}\text{R}^{21}$, $\text{C(S)}\text{OR}^{22}$, and substituted and
25 non-substituted aryl and substituted and non-substituted heteroaryl which, if substituted,
carry at least one substituent from the group consisting of $\text{C}_1\text{-C}_3$ -alkyl, $\text{C}_1\text{-C}_3$ -alkoxy,
halogens, pseudohalogens, and CF_3 ;

R^3 , R^4 are independently selected from the group consisting of:
30 H; substituted and unsubstituted methyl and ethyl which, if substituted, can carry one or
more substituents from the group OH, halogens, pseudohalogens,;

R^5 independently has the same meaning as R^3 ;

35 R^6 and R^7 independently have the same meaning as R^3 , R^4 ;

R^8 is H or C_1 - C_3 -alkyl which can be unsubstituted or carry one or more substituents from the group consisting of OH, $C(O)H$, $C(O)CH_3$, $C(O)C_2H_5$, halogens, pseudohalogens, NH_2 , mono(C_1 - C_3 -alkyl)amino, di(C_1 - C_3 -alkyl)amino;

- 5 R^9 is H or C_1 - C_3 -alkyl which can be unsubstituted or carry one or more substituents from the group consisting of OH, $C(O)H$, $C(O)CH_3$, $C(O)C_2H_5$, halogens, pseudohalogens, NH_2 , mono(C_1 - C_3 -alkyl)amino, di(C_1 - C_3 -alkyl)amino;

R^{10} , R^{11} independently have the same meaning as R^3 , R^4 ;

10

R^{12} , R^{13} independently are H or unsubstituted and at least monosubstituted C_1 - C_{12} -alkyl which can carry in its chain one or more non-adjacent heteroatoms from the group nitrogen and oxygen, and which, if substituted, carry one or more substituents from the group consisting of: OH, $C(O)H$, $C(O)CH_3$, $C(O)C_2H_5$, halogens, pseudohalogens, NH_2 ,
15 mono(C_1 - C_3 -alkyl)amino, di(C_1 - C_3 -alkyl)amino, and unsubstituted and at least monosubstituted aryl and heteroaryl, which, if substituted, carry at least one substituent from the group consisting of C_1 - C_3 -alkyl, C_1 - C_3 -alkoxy, halogens, pseudohalogens, and CF_3 ;

- 20 R^{14} has the same meaning as R^{12}

R^{15} , R^{16} independently have the same meaning as R^{12} , R^{13} ;

R^{17} has the same meaning as R^{12} ;

25

R^{18} has the same meaning as R^{12}

R^{19} , R^{20} independently have the same meaning as R^{12} , R^{13} ;

- 30 R^{21} has the same meaning as R^{12} ;

R^{22} has the same meaning as R^{12} ;

aryl is 5 to 10-membered, mono- or bicyclic aromatic cycle;

35

heteroaryl is a 5 to 10-membered, mono- or bicyclic aromatic heterocycle containing one or more heteroatoms from the group consisting of N, O and S;

m is 1, 2 or 3.

5

Alkyl residues can be linear or branched, acyclic or cyclic. This also applies when they are part of other groups, for example in alkoxy groups, alkoxycarbonyl groups or amino groups, or when they are substituted.

- 10 Examples for alkyl groups are methyl, ethyl, propyl, butyl, pentyl, hexyl, the n-isomers of these residues, isopropyl, isobutyl, isopentyl, sec-butyl, tert-butyl, neopentyl, 3,3-dimethylbutyl. The term alkyl here also expressly includes cycloalkyl residues and cycloalkyl-alkyl-residues (alkyl substituted by cycloalkyl) containing at least three carbon atoms. Examples for such cycloalkyl residues are cyclopropyl, cyclobutyl, cyclopentyl,
15 cyclohexyl. All cycloalkyl groups can be substituted by one or more identical or different (C₁-C₄)-alkyl residues, in particular by methyl. Furthermore, unless stated otherwise, the term alkyl here also includes unsubstituted alkyl residues as well as alkyl residues which are substituted by one or more, for example one, two, three or four, identical or different residues, for example aryl groups. In substituted alkyl residues, for example arylalkyl,
20 hydroxyalkyl such as -(C₁-C₃)-alkyl-OH or alkoxyalkyl such as -(C₁-C₃)-alkyl-O-(C₁-C₄)-alkyl, the substituents can be present in any desired position.

The term methylene group as used in the context of the present invention denotes a -CH₂- unit or a unit derived therefrom by replacing one or both H atom for a substituent.

25

The term aryl as used in the context of the present invention comprises mononuclear, binuclear or trinuclear aryl groups having no heteroatoms in its cycle. If not stated otherwise, the aryl groups can carry one or more substituents for example alkyl groups, alkoxy groups, halogens or OH groups. Examples for aryl groups include phenyl, 1-naphthyl, 2-naphthyl, indanyl, toluyl.

30

Unless stated otherwise, heteroaryl residues which are formed by two groups bonded to a nitrogen are preferably derived from heterocycles which contain one, two, three or four heteroatoms which can be identical or different; more preferably they are derived from
35 heterocycles which contain one, two, or three, in particular one or two, heteroatoms which can be identical or different. Unless stated otherwise, the heterocycles can be mononuclear

or polynuclear, for example mononuclear, binuclear or trinuclear. Preferably they are mononuclear or binuclear. The rings preferably are 5-membered rings, 6-membered rings or 7-membered rings. Examples of mononuclear and binuclear heterocyclic systems from which residues occurring in the compounds of the formula (I) can be derived, are pyrrole, furan, thiophene, imidazole, pyrazole, 1,2,3-triazole, 1,2,4-triazole, 1,3-dioxole, 1,3-oxazole (= oxazole), 1,2-oxazole (= isoxazole), 1,3-thiazole (= thiazole), 1,2-thiazole (= isothiazole), tetrazole, pyridine, pyridazine, pyrimidine, pyrazine, pyran, thiopyran, 1,4-dioxine, 1,2-oxazine, 1,3-oxazine, 1,4-oxazine, 1,2-thiazine, 1,3-thiazine, 1,4-thiazine, 1,2,3-triazine, 1,2,4-triazine, 1,3,5-triazine, 1,2,4,5-tetrazine, azepine, 1,2-diazepine, 1,3-diazepine, 1,4-diazepine, 1,3-oxazepine, 1,3-thiazepine, indole, benzothiophene, benzofuran, benzothiazole, benzimidazole, quinoline, isoquinoline, cinnoline, quinazoline, quinoxaline, phthalazine, thienothiophenes, 1,8-naphthyridine and other naphthyridines, pteridin, or phenothiazine, each of them in saturated form (perhydro form) or in partially unsaturated form (for example in the dihydro form or the tetrahydro form) or in maximally unsaturated form, in case the respective forms are known and stable. Thus, suitable heterocycles also include, for example, the saturated heterocycles pyrrolidine, piperidine, piperazine, morpholine and thiomorpholine. The degree of saturation of heterocyclic groups is indicated in their individual definitions. Unsaturated heterocycles can contain, for example, one, two or three double bonds within the ring system. 5-membered rings and 6-membered rings can in particular also be aromatic.

Halogen is fluorine, chlorine, bromine or iodine, preferably fluorine or chlorine.

Examples for pseudohalogens are CN and N₃, a preferred pseudohalogen is CN.

Preferred compounds of the formula (I) are those compounds in which one or more of the residues contained therein have the meanings given in the following, with all combinations of preferred substituent definitions being a subject of the present invention. With respect to all preferred compounds of the formula (I) the present invention also includes all stereoisomeric forms and mixtures thereof in all ratios, and their pharmaceutically acceptable salts.

Compounds of the formula (I) in which all of the above-mentioned groups have the preferred meanings or the particularly preferred meanings are more preferred with respect to the compounds of formula (I) wherein only one or several of the substituents have the preferred or more preferred meanings.

R^1 and R^2 are independently from each other selected from the group consisting of:

H; OH; (=O); halogens; pseudohalogens; NH_2 ; $S(O)_mR^5$; $S(O)_2NH_2$; $C(O)R^8$; $C(O)OR^9$; $CONH_2$; C_1 - C_2 -alkyl substituted by NH_2 , OH, $S(O)_mR^5$, $S(O)_2NH_2$, $C(O)R^8$, $C(O)OR^9$, $CONH_2$; C_1 - C_2 -alkoxy substituted by NH_2 , OH, $S(O)_mR^5$, $S(O)_2NH_2$, $C(O)R^8$, $C(O)OR^9$, $CONH_2$;

Ar denotes an unsubstituted mononuclear aryl group having 6 or 7 members, which aryl group is annulated to the neighbouring 5-membered cycle, and which may carry 1, 2 or 3 heteroatoms from the group N, O and S in its cycle;

Y, Z denote independently from each other a nitrogen atom or a methylene group;

X is a nitrogen atom or a methylene group;

15

A is selected from the group consisting of: H; halogens and pseudohalogens; OH; $=N(OH)$; $NR^{12}R^{13}$; OSO_3^- ; $S(O)_mR^{14}$; $SO_2NR^{15}R^{16}$; $C(O)R^{17}$; $C(O)OR^{18}$; $CONR^{19}R^{20}$; $C(S)R^{21}$; $C(S)OR^{22}$; unsubstituted and at least monosubstituted C_1 - C_6 -alkyl which can carry in its chain one or more non-adjacent heteroatoms from the group nitrogen and oxygen, and which, if substituted, carry at least one substituent which is preferably selected from the group consisting of: halogens, pseudohalogens, OH, $NR^{12}R^{13}$, OSO_3^- , $S(O)_mR^{14}$, $SO_2NR^{15}R^{16}$, $C(O)R^{17}$, $C(O)OR^{18}$, $CONR^{19}R^{20}$, $C(S)R^{21}$, $C(S)OR^{22}$, and substituted and non-substituted aryl and substituted and non-substituted heteroaryl which, if substituted, carry at least one substituent from the group consisting of C_1 - C_3 -alkyl, C_1 - C_3 -alkoxy, halogens, pseudohalogens, and CF_3 ;

25

R^5 is selected from H; substituted and unsubstituted methyl and ethyl which, if substituted, can carry one or more substituents from the group OH, halogens, pseudohalogens,;

R^8 is H or C_1 - C_3 -alkyl which can be unsubstituted or carry one or more substituents from the group consisting of OH, $C(O)H$, $C(O)CH_3$, $C(O)C_2H_5$, halogens, pseudohalogens; NH_2 , mono(C_1 - C_3 -alkyl)amino, di(C_1 - C_3 -alkyl)amino;

30

R^9 is H or C_1 - C_3 -alkyl which can be unsubstituted or carry one or more substituents from the group consisting of OH, $C(O)H$, $C(O)CH_3$, $C(O)C_2H_5$, halogens, pseudohalogens; NH_2 , mono(C_1 - C_3 -alkyl)amino, di(C_1 - C_3 -alkyl)amino;

35

R^{12} , R^{13} independently are H or unsubstituted and at least monosubstituted C_1 - C_{12} -alkyl which can carry in its chain one or more non-adjacent heteroatoms from the group nitrogen and oxygen, and which, if substituted, carry one or more substituents from the group consisting of: OH, $C(O)H$, $C(O)CH_3$, $C(O)C_2H_5$, halogens, pseudohalogens, NH_2 , mono(C_1 - C_3 -alkyl)amino, di(C_1 - C_3 -alkyl)amino, and unsubstituted and at least monosubstituted aryl and heteroaryl, which, if substituted, carry at least one substituent from the group consisting of C_1 - C_3 -alkyl, C_1 - C_3 -alkoxy, halogens, pseudohalogens, and CF_3 ;

R^{14} has the same meaning as R^{12}

R^{15} , R^{16} independently have the same meaning as R^{12} , R^{13} ;

R^{17} has the same meaning as R^{12} ;

R^{18} has the same meaning as R^{12}

R^{19} , R^{20} independently have the same meaning as R^{12} , R^{13} ;

R^{21} has the same meaning as R^{12} ;

R^{22} has the same meaning as R^{12} ;

aryl is phenyl, naphth-1-yl or naphth-2-yl.

heteroaryl is preferably selected from the group consisting of 5- and 6- membered monocyclic and 9- or 10-membered bicyclic heterocycles containing one or more heteroatoms from the group consisting of N, O, and S; heteroaryl is in particular selected from the group consisting of indolyl, furyl, pyrrolyl, thienyl, thiazolyl, oxazolyl, pyrazolyl, imidazolyl, pyrazinyl, pyridyl and pyrimidinyl.;

m is 1, 2 or 3.

In a further preferred embodiment of the present invention, at least one substituent R^1 or R^2 in formula (I) is (=O).

In a further preferred embodiment of the present invention, in case A is a substituted alkyl group, the carbon atom in α -position to X carries at least 1 H-atom.

5 It is even more preferred if the substituents in formula I have following meanings :

R^1 and R^2 are independently from each other selected from the group consisting of:
H; OH; (=O); halogens; pseudohalogens; with at least one of R^1 , R^2 being (=O);

10 Ar denotes an unsubstituted mononuclear aryl group having 6 or 7 members, which aryl group is annulated to the neighbouring 5-membered cycle, and which may carry 1 or 2 heteroatoms from the group N, O and S in its cycle;

Y, Z denote independently from each other a nitrogen atom or a methylene group;

15

X is a nitrogen atom or a methylene group;

A is an at least monosubstituted C_1 - C_3 -alkyl having a H-atom in position α to X, which alkyl can carry in its chain one or more non-adjacent heteroatoms from the group nitrogen and oxygen, wherein the at least one substituent is selected from the group consisting of:
20 $C(O)R^{17}$, $C(O)OR^{18}$, and substituted and non-substituted aryl and substituted and non-substituted heteroaryl which aryl and heteroaryl, if substituted, carry at least one substituent from the group consisting of C_1 - C_3 -alkyl, C_1 - C_3 -alkoxy, halogens, pseudohalogens, and CF_3 ;

25

R^{17} is selected from H and unsubstituted C_1 - C_3 -alkyl

R^{18} has the same meaning as R^{17}

30 aryl is phenyl, naphth-1-yl or naphth-2-yl.

heteroaryl is selected from the group consisting of indolyl, furyl, pyrrolyl, thienyl, thiazolyl, oxazolyl, pyrazolyl, imidazolyl, pyrazinyl, pyridyl and pyrimidinyl.

35 In one embodiment of the present invention, it is even still more preferred if the substituents in formula I have the following meanings:

R^1 and R^2 are independently from each other selected from the group consisting of:
H; (=O); with at least one of R^1 , R^2 being (=O), preferably both being (=O);

5 Ar denotes an unsubstituted mononuclear aryl group having 6 or 7 members, which aryl group is annulated to the neighbouring 5-membered cycle, and which may carry 1 or 2 nitrogen atoms in its cycle;

10 Y, Z denote independently from each other a nitrogen atom or a methylene group, preferably Y, Z are both methylene;

X is a nitrogen atom or a methylene group, preferably X is a nitrogen atom;

15 A is an at least bisubstituted C_1 - C_3 -alkyl having a H-atom in position α to X, wherein the at least two substituents are selected from the group consisting of: $C(O)OR^{18}$, and substituted and non-substituted aryl and substituted and non-substituted heteroaryl which aryl and heteroaryl, if substituted, carry at least one substituent from the group consisting of C_1 - C_3 -alkyl, C_1 - C_3 -alkoxy, halogens, pseudohalogens, and CF_3 ;

20 R^{18} is selected from H and unsubstituted C_1 - C_3 -alkyl

aryl is phenyl, naphth-1-yl or naphth-2-yl.

25 heteroaryl is selected from the group consisting of indolyl, furyl, pyrrolyl, thienyl, thiazolyl, oxazolyl, pyrazolyl, imidazolyl, pyrazinyl, pyridyl and pyrimidinyl.

Within this embodiment of the present invention, it is most preferred if the substituents in formula I have the following meanings:

30 R^1 and R^2 are both (=O);

35 Ar denotes an unsubstituted mononuclear aryl group having 6 or 7 members, which aryl group is annulated to the neighbouring 5-membered cycle, and which may carry 1 nitrogen atom in its cycle;

Y, Z are both methylene;

X is a nitrogen atom;

- 5 A is a bisubstituted C₁-C₃-alkyl having a H-atom in position α to X, wherein one substituent is selected from the group consisting of C(O)OR¹⁸, and the other substituent is selected from substituted and non-substituted aryl and substituted and non-substituted heteroaryl which aryl and heteroaryl, if substituted, carry at least one substituent from the group consisting of C₁-C₃-alkyl, C₁-C₃-alkoxy, halogens, pseudohalogens, and CF₃;

10

R¹⁸ is selected from H and unsubstituted C₁-C₃-alkyl;

aryl is phenyl, naphth-1-yl or naphth-2-yl.

- 15 heteroaryl is indolyl.

In another embodiment of the present invention, it is even still more preferred if the substituents in formula I have the following meanings:

- 20 R¹ and R² are independently from each other selected from the group consisting of: H; (=O); with at least one of R¹, R² being (=O), preferably both being (=O);

- Ar denotes an unsubstituted mononuclear aryl group having 6 or 7 members, which aryl group is annulated to the neighbouring 5-membered cycle, and which may carry 1 or 2
25 nitrogen atoms in its cycle;

Y, Z denote independently from each other a nitrogen atom or a methylene group, preferably Y, Z are both methylene;

- 30 X is a nitrogen atom or a methylene group, preferably X is a nitrogen atom;

A is an at least monosubstituted C₁-C₃-alkyl having a H-atom in position α to X, wherein the at least one substituent is selected from the group consisting of: C(O)OR¹⁸, C(S)OR²²;

- 35 R¹⁸ is selected from H and unsubstituted C₁-C₃-alkyl;

R²² is selected from H and unsubstituted C₁-C₃-alkyl.

Within this embodiment of the present invention, it is most preferred if the substituents in formula I have the following meanings:

R¹ and R² are both (=O);

Ar denotes an unsubstituted mononuclear aryl group having 6 or 7 members, which aryl group is annulated to the neighbouring 5-membered cycle, and which may carry 1 nitrogen atom in its cycle;

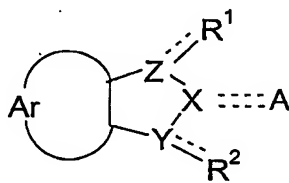
Y, Z are both methylene;

X is a nitrogen atom;

A is a monosubstituted C₁-C₃-alkyl having a H-atom in position α to X, wherein the substituent is C(O)OR¹⁸;

R¹⁸ is selected from H and unsubstituted C₁-C₃-alkyl.

The object of the present invention is also attained by compounds according to the general formula (I)



(I)

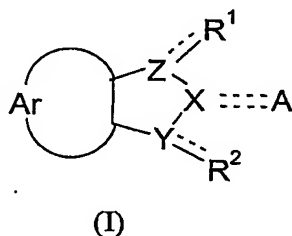
wherein the symbols Ar, A, X, Y, and Z and the substituents R¹ and R² have the meanings defined above for all normal and preferred embodiments, for use as a pharmaceutical.

Preferred compounds of the formula (I) for use as a pharmaceutical are those compounds in which one or more of the residues contained therein have the meanings given above as being preferred, with all combinations of preferred substituent definitions being a subject of the present invention. With respect to all preferred compounds of the formula (I) for use

as a pharmaceutical, the present invention also includes all stereoisomeric forms and mixtures thereof in all ratios, and their pharmaceutically acceptable salts.

Compounds of the formula (I) in which all of the above-mentioned groups have the preferred meanings or the particularly preferred meanings are more preferred for use as a pharmaceutical with respect to the compounds of formula (I) wherein only one or several of the substituents have the preferred or more preferred meanings.

The object of the present invention is furthermore attained by the use of a compound of formula (I)

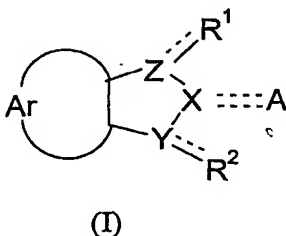


wherein the symbols Ar, A, X, Y, and Z and the substituents R¹ and R² have the meanings defined above for all normal and preferred embodiments, for the manufacture of a pharmaceutical for the inhibition of DNMTs, more particularly DNMT1, and/or the inhibition of DNA methylation, and the pharmaceuticals obtained accordingly.

Preferred compounds of the formula (I) which can be used for the manufacture of the said pharmaceuticals are those compounds in which one or more of the residues contained therein have the meanings given above as being preferred, with all combinations of preferred substituent definitions being a subject of the present invention. With respect to all preferred compounds of the formula (I) which can be used for the manufacture of the said pharmaceuticals are, the present invention also includes all stereoisomeric forms and mixtures thereof in all ratios, and their pharmaceutically acceptable salts.

Compounds of the formula (I) in which all of the above-mentioned groups have the preferred meanings or the particularly preferred meanings are more preferred for the manufacture of the said pharmaceuticals with respect to the compounds of formula (I) wherein only one or several of the substituents have the preferred or more preferred meanings.

The present invention also relates to the use compounds according to formula (I)



wherein the symbols Ar, A, X, Y, and Z and the substituents R¹ and R² have the meanings defined above for all normal and preferred embodiments, in methods of treatment.

The present invention includes all stereoisomeric forms of the compounds of the formula (I). Centers of asymmetry that are present in the compounds of formula (I) all independently of one another have S configuration or R configuration. The invention includes all possible enantiomers and diastereomers and mixtures of two or more stereoisomers, for example mixtures of enantiomers and/or diastereomers, in all ratios. Thus, compounds according to the present invention which can exist as enantiomers can be present in enantiomerically pure form, both as levorotatory and as dextrorotatory antipodes, in the form of racemates and in the form of mixtures of the two enantiomers in all ratios. In the case of a cis/trans isomerism the invention includes both the cis form and the trans form as well as mixtures of these forms in all ratios. All these forms are an object of the present invention. The preparation of individual stereoisomers can be carried out, if desired, by separation of a mixture by customary methods, for example by chromatography or crystallization, by the use of stereochemically uniform starting materials for the synthesis or by stereoselective synthesis. Optionally a derivatization can be carried out before a separation of stereoisomers. The separation of a mixture of stereoisomers can be carried out at the stage of the compounds of the formula (I) or at the stage of an intermediate during the synthesis. The present invention also includes all tautomeric forms of the compounds of formula (I).

In case the compounds according to formula (I) contain one or more acidic or basic groups, the invention also comprises their corresponding pharmaceutically or toxicologically acceptable salts, in particular their pharmaceutically utilizable salts. Thus, the compounds of the formula (I) which contain acidic groups can be present on these groups and can be used according to the invention, for example, as alkali metal salts, alkaline earth metal salts or as ammonium salts. More precise examples of such salts include sodium salts,

potassium salts, calcium salts, magnesium salts or salts with ammonia or organic amines such as, for example, ethylamine, ethanolamine, triethanolamine or amino acids. Compounds of the formula (I) which contain one or more basic groups, i.e. groups which can be protonated, can be present and can be used according to the invention in the form of their addition salts with inorganic or organic acids. Examples for suitable acids include hydrogen chloride, hydrogen bromide, phosphoric acid, sulfuric acid, nitric acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acids, oxalic acid, acetic acid, tartaric acid, lactic acid, salicylic acid, benzoic acid, formic acid, propionic acid, pivalic acid, diethylacetic acid, malonic acid, succinic acid, pimelic acid, fumaric acid, maleic acid, malic acid, sulfaminic acid, phenylpropionic acid, gluconic acid, ascorbic acid, isonicotinic acid, citric acid, adipic acid, and other acids known to the person skilled in the art. If the compounds of the formula (I) simultaneously contain acidic and basic groups in the molecule, the invention also includes, in addition to the salt forms mentioned, inner salts or betaines (zwitterions). The respective salts according to the formula (I) can be obtained by customary methods which are known to the person skilled in the art like, for example by contacting these with an organic or inorganic acid or base in a solvent or dispersant, or by anion exchange or cation exchange with other salts. The present invention also includes all salts of the compounds of the formula (I) which, owing to low physiological compatibility, are not directly suitable for use in pharmaceuticals but which can be used, for example, as intermediates for chemical reactions or for the preparation of pharmaceutically acceptable salts.

The present invention furthermore includes all solvates of compounds of the formula (I), for example hydrates or adducts with alcohols, active metabolites of the compounds of the formula (II), and also derivatives and prodrugs of the compounds of the formula (I) which contain physiologically tolerable and cleavable groups, for example esters, amides and compounds in which the N-H group depicted in formula (I) is replaced with an N-alkyl group, such as N-methyl, or with an N-acyl group, such as N-acetyl or N-argininyl, including pharmaceutically acceptable salts formed on functional groups present in the N-acyl group.

The compounds according to general formula (I) and their precursors can be prepared according to methods published in the literature or, respectively, analogous methods. Appropriate methods have been published in, for example, Houben-Weyl, Methoden der Organischen Chemie (Methods of Organic Chemistry), Thieme-Verlag, Stuttgart, or Organic Reactions, John Wiley & Sons, New York.

All reactions for the synthesis of the compounds of the formula (I) are per se well-known to the skilled person and can be carried out under standard conditions according to or analogously to procedures described in the literature, for example in Houben-Weyl, Methoden der Organischen Chemie (Methods of Organic Chemistry), Thieme-Verlag, Stuttgart, or Organic Reactions, John Wiley & Sons, New York. Depending on the circumstances of the individual case, in order to avoid side reactions during the synthesis of a compound of the formula (I), it can be necessary or advantageous to temporarily block functional groups by introducing protective groups and to deprotect them in a later stage of the synthesis, or introduce functional groups in the form of precursor groups which in a later reaction step are converted into the desired functional groups. Such synthesis strategies and protective groups and precursor groups which are suitable in an individual case are known to the skilled person. If desired, the compounds of the formula (I) can be purified by customary purification procedures, for example by recrystallization or chromatography. The starting compounds for the preparation of the compounds of the formula (I) are commercially available or can be prepared according to or analogously to literature procedures. The compounds obtained with the above-mentioned synthesis methods are a further object of the present invention.

A part of the compounds falling under formula (I) are disclosed in the literature. However, their use in inhibition of DNA methylation or the treatment of developmental or proliferative disorders is not disclosed in any of these references.

The compounds according to the general formula (I) can be used to inhibit DNA methylation in cells. Particularly, said compounds inhibit DNMTs, more particularly DNMT1, even more particularly human DNMT1.

As the compounds according to the general formula (I) are inhibitors of DNA methylation, they are helpful pharmaceutical compounds for the treatment of diseases associated with aberrant DNA methylation or treatable by inhibition of DNA methylation. In the context of the present invention, treatment includes the therapy as well as the prophylaxis of the respective diseases.

Aberrant DNA methylation preferably relates to any kind of hypermethylation, be it genome-wide or limited to distinct genomic or chromosomal regions or genes. DNA methylation can be measured by any of the methods known in the art (e.g. Okamoto, A., et

al. (2002). Site-specific discrimination of cytosine and 5-methylcytosine in duplex DNA by peptide nucleic acids. *J Am. Chem. Soc.* 124, 10262-10263), methylation sensitive arbitrarily primed PCR (Gonzalzo, M.L., Liang, G., et al. (1997). Identification and characterization of differentially methylated regions of genomic DNA by methylation-sensitive arbitrarily primed PCR. *Cancer Res.* 57:594-599), methylated CpG island amplification (Toyota, M., Ho, C., et al. (1999). Identification of differentially methylated sequences in colorectal cancer by methylated CpG island amplification. *Cancer Res.* 59:2307-2312), restriction landmark genomic scanning (RLGS) (Hayashizaki, Y., Hirotsune, S., et al., (1993). Restriction landmark genomic scanning method and its various applications. *Electrophoresis* 14:251-258), differential methylation hybridization (Huang, T. H., Perry, M. R., et al. (1999). Methylation profiling of CpG islands in human breast cancer cells. *Hum. Mol. Genet.* 8: 459-470), capillary electrophoresis (Stach, D., Schmitz, O.J., Stilgenbauer, S., et al. 2003. *Nucleic Acids Res.* 31, e2), and microarray-based techniques (Adorján, P., Distler, J., et al. (2002). Tumour class prediction and discovery by microarray-based DNA methylation analysis. *Nucleic Acids Research* 30(5): e21). Thus, it is also possible to identify diseases associated with aberrant DNA methylation.

Examples of diseases which can be treated with the compounds according to the present invention include developmental disorders and proliferative diseases.

Examples for developmental disorders which can be treated with the compounds according to the present invention include Prader-Willi-Syndrome, Angelman-Syndrome (Happy Puppet Syndrome), and Beckwith-Wiedemann-Syndrome.

Examples for proliferative diseases which can be treated with the compounds according to the present invention include coronary restenosis and neoplastic diseases.

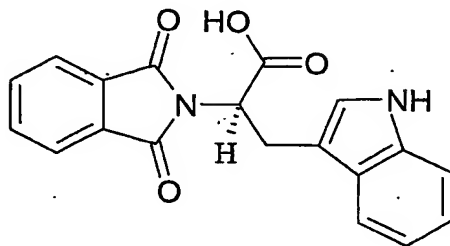
Said neoplastic diseases include neuroblastoma, intestine carcinoma such as rectum carcinoma, colon carcinoma, familial adenomatous polyposis carcinoma and hereditary non-polyposis colorectal cancer, esophageal carcinoma, labial carcinoma, larynx carcinoma, hypopharynx carcinoma, tongue carcinoma, salivary gland carcinoma, gastric carcinoma, adenocarcinoma, medullary thyroid carcinoma, papillary thyroid carcinoma, renal carcinoma, kidney parenchym carcinoma, ovarian carcinoma, cervix carcinoma, uterine corpus carcinoma, endometrium carcinoma, chorion carcinoma, pancreatic carcinoma, prostate carcinoma, testis carcinoma, breast carcinoma, urinary

carcinoma, melanoma, brain tumors such as glioblastoma, astrocytoma, meningioma, medulloblastoma and peripheral neuroectodermal tumors, Hodgkin lymphoma, non-Hodgkin lymphoma, Burkitt lymphoma, acute lymphatic leukemia (ALL), chronic lymphatic leukemia (CLL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML), adult T-cell leukemia lymphoma, hepatocellular carcinoma, gall bladder carcinoma, bronchial carcinoma, small cell lung carcinoma, non-small cell lung carcinoma, multiple myeloma, basalioma, teratoma, retinoblastoma, choroidea melanoma, seminoma, rhabdomyosarcoma, craniopharyngeoma, osteosarcoma, chondrosarcoma, myosarcoma, liposarcoma, fibrosarcoma, Ewing sarcoma, prostate carcinoma, and plasmocytoma.

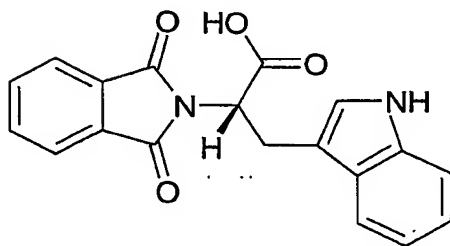
Preferred indications are colon carcinoma, familial adenomatous polyposis carcinoma and hereditary non-polyposis colorectal cancer, prostate carcinoma, melanoma, non-Hodgkin lymphoma, acute lymphatic leukemia (ALL), chronic lymphatic leukemia (CLL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML), or hepatocellular carcinoma.

The compounds according to the formula (I) can also be used in combination with other pharmaceutically active compounds, preferably compounds which are able to enhance the effect of the compounds according to the general formula (I). Examples of such compounds include: (i) antimetabolites, cytarabine, fludarabine, 5-fluoro-2'-deoxyuridine, gemcitabine, hydroxyurea or methotrexate; (ii) DNA-fragmenting agents, bleomycin, (iii) DNA-crosslinking agents, chlorambucil, cisplatin, fotemustine, cyclophosphamide or nitrogen mustard; (iv) intercalating agents, adriamycin (doxorubicin) or mitoxantrone; (v) protein synthesis inhibitors, L-asparaginase, cycloheximide, puromycin or diphtheria toxin; (vi) topoisomerase I poisons, camptothecin or topotecan; (vii) topoisomerase II poisons, etoposide (VP-16) or teniposide; (viii) microtubule-directed agents, colcemid, colchicine, paclitaxel (taxol), docetaxel (taxotere), vinblastine or vincristine; (ix) kinase inhibitors, flavopiridol, staurosporin, STI571 (CPG 57148B) or UCN-01 (7-hydroxystaurosporine); (x) miscellaneous investigational agents, trichostatin A, thioplatin, PS-341, phenylbutyrate, ET-18-OCH₃, or farnesyl transferase inhibitors (L-739749, L-744832); polyphenols, quercetin, resveratrol, piceatannol, epigallocatechine gallate, theaflavins, flavanols, procyanidins, betulinic acid and derivatives thereof; (xi) hormones, glucocorticoids or fenretinide; (xii) hormone antagonists, tamoxifen, finasteride or LHRH antagonists, (xiii) demethylating agents, 5-azacytidine, 5-aza-2'-deoxycytidine, 5,6-dihydro-5-azacytidine, or (xiv) a combination of any of the pharmaceuticals given above.

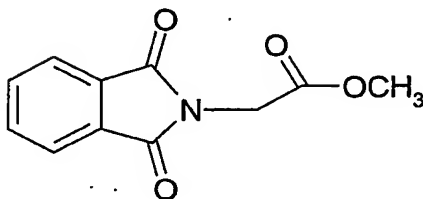
Preferred compounds are the compounds according to the formulae below.



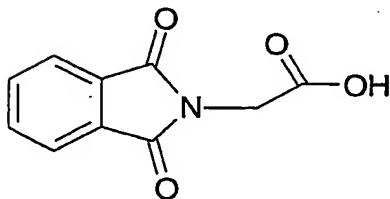
(II) RG108



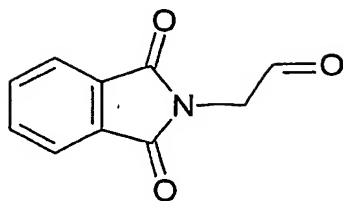
(III)



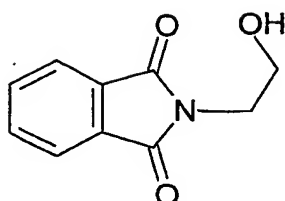
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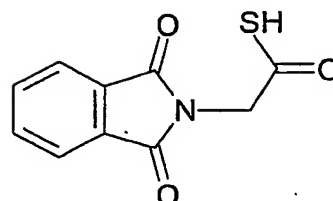
(V)



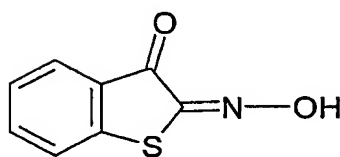
(VI)



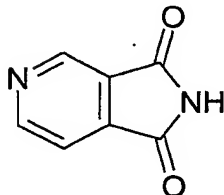
(VII)



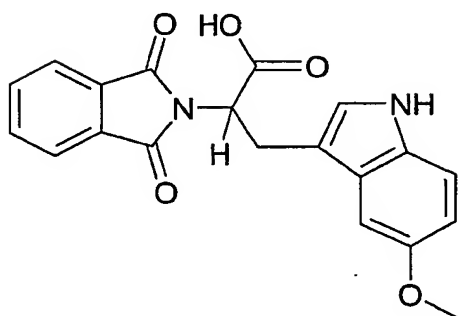
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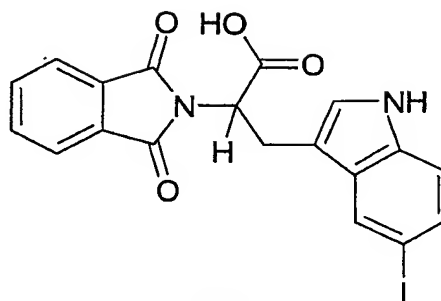
(IX)



(X)



(XI)



(XII)

- 10 The compounds of the formula (I) and their pharmaceutically acceptable salts, optionally in combination with other pharmaceutically active compounds, can be administered to animals, preferably to mammals, and in particular to humans, as pharmaceuticals by themselves, in mixtures with one another or in the form of pharmaceutical preparations. Further subjects of the present invention therefore also are the compounds of the formula
- 15 (I) and their pharmaceutically acceptable salts for use as pharmaceuticals, their use as inhibitors of DNMTs and/or DNA methylation, and in particular their use in the therapy and prophylaxis of the above-mentioned syndromes as well as their use for preparing pharmaceuticals for these purposes. Furthermore, subjects of the present invention are pharmaceutical preparations (or pharmaceutical compositions) which comprise an effective
- 20 dose of at least one compound of the formula (I) and/or a pharmaceutically acceptable salt thereof and a pharmaceutically acceptable carrier, i.e. one or more pharmaceutically acceptable carrier substances and/or additives.

The pharmaceuticals according to the invention can be administered orally, for example in the form of pills, tablets, lacquered tablets, sugar-coated tablets, granules, hard and soft gelatin capsules, aqueous, alcoholic or oily solutions, syrups, emulsions or suspensions, or rectally, for example in the form of suppositories. Administration can also be carried out parenterally, for example subcutaneously, intramuscularly or intravenously in the form of solutions for injection or infusion. Other suitable administration forms are, for example, percutaneous or topical administration, for example in the form of ointments, tinctures, sprays or transdermal therapeutic systems, or the inhalative administration in the form of nasal sprays or aerosol mixtures, or, for example, microcapsules, implants or rods. The preferred administration form depends, for example, on the disease to be treated and on its severity.

The amount of compounds of the formula (I) and/or its pharmaceutically acceptable salts in the pharmaceutical preparations normally ranges from 0.2 to 800 mg, preferably from 0.5 to 500 mg, in particular from 1 to 200 mg, per dose, but depending on the type of the pharmaceutical preparation it may also be higher. The pharmaceutical preparations usually comprise 0.5 to 90 percent by weight of the compounds of the formula (I) and/or their pharmaceutically acceptable salts. The preparation of the pharmaceutical preparations can be carried out in a manner known per se. To this end, one or more compounds of the formula (I) and/or their pharmaceutically acceptable salts, together with one or more solid or liquid pharmaceutical carrier substances and/or additives (or auxiliary substances) and, if desired, in combination with other pharmaceutically active compounds having therapeutic or prophylactic action, are brought into a suitable administration form or dosage form which can then be used as a pharmaceutical in human or veterinary medicine.

For the production of pills, tablets, sugar-coated tablets and hard gelatin capsules it is possible to use, for example, lactose, starch, for example maize starch, or starch derivatives, talc, stearic acid or its salts, etc. Carriers for soft gelatin capsules and suppositories are, for example, fats, waxes, semisolid and liquid polyols, natural or hardened oils, etc. Suitable carriers for the preparation of solutions, for example of solutions for injection, or of emulsions or syrups are, for example, water, physiologically sodium chloride solution, alcohols such as ethanol, glycerol, polyols, sucrose, invert sugar, glucose, mannitol, vegetable oils, etc. It is also possible to lyophilize the compounds of the formula (I) and their pharmaceutically acceptable salts and to use the resulting lyophilisates, for example, for preparing preparations for injection or infusion. Suitable

carriers for microcapsules, implants or rods are, for example, copolymers of glycolic acid and lactic acid.

Besides the compound or compounds according to the invention and carriers, the pharmaceutical preparations can also contain additives, for example fillers, disintegrants, binders, lubricants, wetting agents, stabilizers, emulsifiers, dispersants, preservatives, sweeteners, colorants, flavorings, aromatizers, thickeners, diluents, buffer substances, solvents, solubilizers, agents for achieving a depot effect, salts for altering the osmotic pressure, coating agents or antioxidants.

10

The dosage of the compound of the formula (I) to be administered and/or of a pharmaceutically acceptable salt thereof depends on the individual case and is, as is customary, to be adapted to the individual circumstances to achieve an optimum effect. Thus, it depends on the nature and the severity of the disorder to be treated, and also on the sex, age, weight and individual responsiveness of the human or animal to be treated, on the efficacy and duration of action of the compounds used, on whether the therapy is acute or chronic or prophylactic, or on whether other active compounds are administered in addition to compounds of the formula (I). In general, a daily dose of approximately 0.01 to 100 mg/kg, preferably 0.1 to 10 mg/kg, in particular 0.3 to 5 mg/kg (in each case mg per kg of bodyweight) is appropriate for administration to an adult weighing approximately 75 kg in order to obtain the desired results. The daily dose can be administered in a single dose or, in particular when larger amounts are administered, be divided into several, for example two, three or four individual doses. In some cases, depending on the individual response, it may be necessary to deviate upwards or downwards from the given daily dose.

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The compounds of the formula (I) may also be used to induce cellular differentiation in vivo and in vitro. Cellular differentiation relates to any differentiation of a cell from a less differentiated (specialized) state to a more differentiated (specialized) state. Cell types which can be treated include, but are not limited to, embryonic and adult stem cells, totipotent, omnipotent, pluripotent, multipotent, oligopotent, or monopotent stem cells, progenitor cells, committed progenitor cells, as well as stem cells derived from bone marrow, peripheral blood, umbilical cord blood, adipose tissue, heart muscle, intestine, small intestine, or brain. Particular examples include multipotent adult progenitor cells (MAPCs), mesenchymal stem cells, hematopoietic stem cells, intestinal stem cells, hepatic stem cells (oval cells), neuronal stem cells, epidermal stem cells, myoblasts,

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cardiomyoblasts, osteoblasts, chondroblasts, and basal cells of epithelia, e.g. the respiratory epithelium.

5 The compounds according to formula (I) are capable of binding to DNMTs, particularly human DNMT1, and inhibiting their catalytic activity.

The GenBank accession numbers of selected DNMTs of human, mouse, *Drosophila melanogaster*, *Haemophilus haemolyticus* and *Haemophilus aegyptius* are given below:

10 human DNMT1 protein (GenBank Acc. No. NP_001370)
human DNMT2 protein (GenBank Acc. No. AAC39764)
human DNMT3A protein (GenBank Acc. No. AAD33084)
human DNMT3B protein (GenBank Acc. No. AAD53063)
mouse DNMT1 protein (GenBank Acc. No. NP_034196)
15 *D. melanogaster* dDNMT2 protein (GenBank Acc. No. AAF03835)
H. haemolyticus M.HhaI methyltransferase protein (GenBank Acc. No. XYHIH1)
H. aegyptius M.HhaI methyltransferase protein (GenBank Acc. No. AAA24970)

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The present invention explicitly includes the use of the compounds according to formula (I) for inhibition of DNA methylation or inhibition of DNMTs in cells of prokaryotes, eukaryotes, invertebrates, vertebrates, particularly mammals, more particularly rodents and primates, even more particularly humans.

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Different DNMTs in different species are highly conserved, i.e. structurally similar, particularly with respect to their C-terminal catalytic domains (see SEQ ID No. 1-8). Therefore, the compounds according to formula (I) are capable of binding and inhibiting different DNMTs in different species.

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One advantage of the compounds according to the present invention is that they are able to substantially demethylate and reactivate euchromatic genes (e.g. tumor suppressor genes), but not centromeric satellite sequences. This is an advantage for treatment of cells or patients, as demethylation of satellite sequences has been shown to promote tumorigenesis
35 by destabilizing chromosome organization. This will have a positive effect on maintenance

of genome stability in cells or patients treated with the compounds according to the present invention.

Figure legends

Fig. 1

Calculated binding energies of NSC401077 and various control molecules docked into the DNMT1 active site (a). The calculated binding energy of NSC401077 exceeds that of the natural substrate, cytidine. (b) Structure of RG108. The compound was synthesized as an L-enantiomer, while NSC401077 represents a racemic mixture of both enantiomers.

Fig. 2

Direct inhibition of DNA methyltransferase activity by RG108. (a) In vitro methylation assay. Equal amounts of purified SssI methylase were incubated with a DNA fragment derived from the human p16/CDKN2A promoter and 200 μ M RG108. This resulted in a detectable decrease in DNA methyltransferase activity, as visualized by the appearance of small *Bst*UI restriction fragments. No inhibitory effect was observed with equal concentrations of 5-azacytidine. (b) Increasing amounts of RG108 progressively inhibit methyltransferase activity. RG108 was used at 0, 10, 200, and 500 μ M concentrations, SssI methylase was present at 5 μ M concentration. (c) Trapping assay. HCT116 cells were incubated with equal concentrations of RG108, 5-azacytidine, or no inhibitor. Protein extracts were then probed for the presence of DNMT1, DNMT3B and Actin by Western blotting. This revealed the covalent trapping of DNA methyltransferase proteins by 5-azacytidine. This effect was not observed with RG108.

Fig. 3

The effect of RG108 on human cell lines. (a) Growth of HCT116 (left panel) and NALM-6 cells (right panel) in medium supplemented with 10 μ M RG 108 (filled circles) or 10 μ M 5-azacytidine (grey diamonds). Controls are shown as open squares. (b) Viability of HCT116 and NALM-6 cells under the same conditions as described in (a). (c) Genomic cytosine methylation levels of HCT116 (left panel) and NALM-6 cells (right panel)) in medium supplemented with 10 μ M RG 108 (black bars) or 10 μ M 5-azacytidine (grey bars). Controls are shown as white bars. DNA methylation levels were determined after 5 and 15 days, as indicated. DNA from NALM-6 cells incubated with 5-azacytidine for 15 days could not be analyzed due to degradation. All results were obtained from multiple experiments. Standard deviations for panels (a) and (b) were negligible.

Fig. 4

The carboxyl-group of RG108 is important for its interaction with DNA methyltransferases. (a) Structure of Δ C-RG108, a control compound that lacks the carboxyl-group of RG-108. (b) Calculated binding energies of Δ C-RG108 (grey bar) docked into the DNMT1 active site are compared to cytidine (white bar) and RG108 (black bar). (c) Genomic cytosine methylation levels of HCT116 cells incubated with Δ C-RG108 (black bar) are compared to methylation levels of corresponding cells incubated with no inhibitor (white bar) or RG108 (grey bar).

Fig. 5

RG108 causes complete demethylation of the human hMLH1 gene in cells treated with 10 micromolar RG108, while no demethylation was observed in control experiments without inhibitor or with 10 micromolar 5-azacytidine (Sigma), respectively (Fig. 5). M indicates amplification products from methylated templates, U indicates amplification from unmethylated control templates.

Fig. 6

(a) Effect of increasing concentrations of RG108 on the proliferation rate of HCT116 cells.

Equal amounts of cells were incubated with different concentrations of RG108 and counted after 5 days. Black bars represent results from 3 independent experiments, standard deviations are indicated by error bars. (b) Reactivation of the *p16^{INK4a}* gene (p16) in HCT116 cells treated with RG108. The upper panel shows the sequence of a wild-type RT-PCR product from cells treated with 10 μ M RG108. Untreated HCT116 cells expressed only the mutant allele of *p16* (lower panel). (c) Effect of RG108 on the methylation and expression of various epigenetically silenced genes in HCT116 cells. Methylation-specific PCR (MSP) was used to analyze the methylation status of *p16^{INK4a}*, *SFRP1* and *TIMP-3* in DNA from cells incubated with 10 μ M RG108. RT-PCT (RTP) was used to determine the expression level in cells incubated with 0, 10, 30 or 100 μ M RG108, as indicated. β -Amyloid (β Am) was used as a loading control. (d) Effect of RG108 on the methylation status of centromeric satellite sequences. HCT116 cells were incubated with variable concentrations of RG108 (RG) or 5-azacytidine (aza), as indicated. The methylation status was analyzed by methylation-sensitive Southern analysis. The size of marker fragments (in kbp) is indicated on the sides of the panels, respectively.

pop.doub. after 5d, number of the cell population doublings after 5 days; ctrl., control; α -sat., α -satellite; sat. 2, satellite 2.

Fig. 7

Bisulfite sequencing analysis of the *TIMP-3* CpG island reveals significant demethylation of CpG dinucleotides in RG108-treated cells ($P < 0.05$, as determined by a *t*-test). Filled circles represent methylated CpG dinucleotides, open circles represent unmethylated CpG dinucleotides.

Fig. 8

(a) Methoxy-RG108, a novel derivative with improved solubility, inhibits DNA methyltransferase activity in vitro. The in vitro methylation assay was performed as described in Example 3. Inhibitor concentrations (in μM) are indicated on top of the right panel. "U" indicates the position of the unmethylated restriction fragment, "M" indicates the position of the methylated restriction fragment. (b) RG126, a novel derivative, inhibits DNA methyltransferase activity in vitro. The in vitro methylation assay was performed as described before, inhibitor concentrations (in μM) are indicated on top of the right panel. "U" indicates the position of the unmethylated restriction fragment, "M" indicates the position of the methylated restriction fragments.

The present invention will now be illustrated in the following examples:

EXAMPLES

Example 1a

Chemical synthesis. RG108 was synthesized in two steps with an overall yield of 90%. The intermediate product, a phthalic acid derivative with a protected and an activated ester group, methyl 2-((succinimidooxy)carbonyl)benzoate (MSB) was obtained according to Casimir, J.R., Guichard, G. & Briand, J.P. Methyl 2-((succinimidooxy)carbonyl)benzoate (MSB): a new, efficient reagent for N-phthaloylation of amino acid and peptide derivatives. *J. Org. Chem.* 67, 3764-3768 (2002) from 2-(methoxycarbonyl)benzoic acid and N-hydroxysuccinimide in 90% yield after crystallisation and drying. The structure was confirmed by mass spectrometry (ESI) and ^1H - and ^{13}C -NMR analysis. RG108 was obtained by the reaction of MSB with L-tryptophan under basic conditions (Na_2CO_3) in water/acetonitrile; acidification with 2N HCl, extraction in ethyl acetate and evaporation of

the solvent with an excellent yield of 100%. The pure yellow powder was analysed by mass spectrometry (ESI) and ^1H - and ^{13}C -NMR to confirm the structure of RG108. For the synthesis of ΔC -RG108, tryptamine (dissolved in acetonitrile) was used instead of L-tryptophan.

- 5 High performance liquid chromatography analysis indicated that RG108 had a half-life time of 10 days in neutral aqueous solutions at 37 °C.

Example 1b

- 10 In an analogous way, the compound XI was prepared, starting from methoxylated L-Tryptophane.

Example 1c

- 15 In an analogous way, the compound XI was prepared, starting from iodated L-Tryptophane.

Example 2

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Analysis of genomic DNA methylation. DNA was isolated from cells using the DNeasy kit from Qiagen. Cytosine methylation levels were determined by capillary electrophoresis, as described previously (Stach, D., Schmitz, O.J., Stilgenbauer, S., et al. 2003. Nucleic Acids Res. 31, e2).

25

Example 3

- The effect of RG108 was analyzed in an in vitro DNA methylation assay. For this assay, the purified recombinant CpG methylase M.SssI was used. This enzyme is distinguished by a robust activity and also shows significant structural similarities with the DNMT1 catalytic domain. A 798 bp PCR fragment from the promoter region of the human p16/CDKN2A gene was used as a substrate and DNA methylation was visualized by digestion with the methylation-sensitive restriction enzyme *Bst*UI. Thus, inhibition of
- 30

DNA methyltransferases can be detected by the appearance of unprotected, smaller restriction fragments (Fig. 2a). A direct comparison of RG108 and 5-azacytidine in this assay revealed a readily detectable inhibitory effect for RG108, but not for 5-azacytidine (Fig. 2a). This result is consistent with the assumption that RG108 is able to inhibit the free DNA methyltransferase, while 5-azacytidine needs to be incorporated into DNA. Additional experiments showed that increasing concentrations of RG108 resulted in increasing amounts of unprotected *Bst*UI restriction fragments (Fig. 2b) and thus confirmed a significant, concentration-dependent inhibition by RG108. Complete inhibition was observed when the molar ratio of RG108 to SssI methylase was roughly 100:1 (Fig. 2b).

Similar inhibitory concentrations could be observed for compound XI.

Example 4

Inhibitors like 5-azacytidine and zebularine, have been shown to covalently trap DNA methyltransferases, which can be visualized by concomitant depletion of the enzymes from cell extracts (Liu, K., Wang, Y.F., Cantemir, C. & Muller, M.T. (2003). Endogenous assays of DNA methyltransferases: Evidence for differential activities of DNMT1, DNMT2, and DNMT3 in mammalian cells *In vivo*. *Mol. Cell. Biol.* **23**, 2709-2719 and Cheng, J.C. et al. (2004). Continuous zebularine treatment effectively sustains demethylation in human bladder cancer cells. *Mol Cell Biol* **24**, 1270-1278.

To characterize the inhibitory mechanism of RG108 human HCT116 cells were incubated with RG108 or an equal concentration of 5-azacytidine and protein extracts were analyzed for the presence of the major DNA methyltransferases, DNMT1 and DNMT3B, and actin (as a control). This demonstrated that DNA methyltransferase levels were not affected by RG108 (Fig. 2c), which suggested that RG108 does not result in covalent trapping of DNA methyltransferases. In contrast, 5-azacytidine resulted in a strong depletion of DNMT1 and a weaker reduction in DNMT3B levels (Fig. 2c). These results are consistent with the direct inhibition observed *in vitro* and provide a major distinction between RG108 and previously known inhibitors of DNA methyltransferases.

Example 5

The effect of RG108 on the growth and viability of human cell lines was characterized. HCT116 was chosen, a colon carcinoma line that has been frequently used for DNA methylation analysis (Brattain, M.G., Fine, W.D., Khaled, F.M., Thompson, J. & Brattain, D.E. Heterogeneity of malignant cells from a human colonic carcinoma. *Cancer Res* **41**, 1751-1756 (1981)), and NALM-6, a leukemic B cell precursor line (Hurwitz, R. et al. Characterization of a leukemic cell line of the pre-B phenotype. *Int. J. Cancer* **23**, 174-180 (1979)). The tissue culture media was supplemented with 10 μ M RG108 and the cells were incubated over 15 days. Unsupplemented media and media supplemented with 10 μ M 5-azacytidine were used for controls. At this concentration, RG108 had no effect on the growth and viability of either cell line (Fig. 3a, b). In contrast, 5-azacytidine showed an intermediate effect on HCT116 cells and appeared to be highly toxic for NALM-6 cells (Fig. 3a, b). To analyze the effect of RG108 on DNA methylation, genomic DNA was isolated from cells and the cytosine methylation level was determined by capillary electrophoresis. This revealed a significant (30 %) demethylation of genomic DNA in HCT116 cells that was similar to the effect seen with equal concentrations of 5-azacytidine (Fig. 3c). Significant demethylation of genomic DNA was also observed in NALM-6 cells, where incubation with RG108 reduced DNA methylation by 15 % (after 5 days) and 60 % (after 15 days), respectively (Fig. 3c). Because of the high toxicity of 5-azacytidine in NALM-6 cells, the corresponding DNA methylation level (5 days, Fig. 3c) needs to be interpreted with caution. After 15 days of incubation in 5-azacytidine containing medium, the DNA from NALM-6 cells appeared to be degraded and was not used for further analysis. In summary, the results suggest that RG108 effectively inhibits DNA methylation in human cell lines. At the same time, incubation with RG108 did not seem to affect the growth and viability of these cells under the conditions used in our experiments.

Example 6

Also derivatives of RG108 with a potentially reduced inhibitory activity were investigated. Δ C-RG108 was synthesized, which is a derivative that lacks the central carboxyl-group (Fig. 4a). Docking of this compound into the DNMT1 active site revealed a strongly reduced binding energy (Fig. 4b). When tested in the in vitro assay, Δ C-RG108 failed to inhibit DNA methylation. Similarly, Δ C-RG108 failed to demethylate genomic DNA of HCT116 and NALM-6 cells (Fig. 4c). The results confirm an important role for the carboxyl-group of RG108 in the interaction with the active site of the enzyme and suggest a considerable specificity in the interaction between RG108 and the DNA methyltransferase active site.

Example 7

The substrate DNA for the in vitro methylation assay was generated by PCR amplification of a 798 bp fragment from the promoter region of the human p16/CDKN2 gene. The methylation reaction contained 350-400 ng substrate DNA (13-15 nM DNA, corresponding to 1.6-1.8 μ M CpGs) in reaction buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂, 1mM dithiothreitol, pH 7.9), 80 μ M S-adenosylmethionine, and 4 U of M.SssI methylase (0.5 μ M, New England Biolabs) in a final volume of 50 μ l. Inhibitors were added in concentrations of 10, 100, 200, and 500 μ M, respectively. Reactions were performed at 37 °C for 2 hours. After completion, the reaction was inactivated at 65 °C for 15 min and the DNA was purified using the QIAquick PCR Purification Kit (Qiagen). 250 ng of purified DNA was digested for 3 h at 60 °C with 30 units of BstUI (New England Biolabs) and analyzed on 2 % agarose gels.

Example 8

Trapping assay: Frozen cell pellets (10^6 - 10^8 cells) were thawed on ice and resuspended in 1 ml ice-cold 1x PBS. After centrifugation (< 1000g) at 4 °C for 5 minutes, the supernatant was removed and discarded. The pellet was resuspended with 100-200 μ l ice-cold lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl (pH 8.0), 2 mM PMSF, and 1 % Igepal) and incubated on ice for 40 minutes. The lysate was centrifuged at 4 °C for 15 minutes with 14,000 rpm. Supernatants were frozen in liquid nitrogen and stored at -80 °C. Equal amounts of protein were then separated on SDS polyacrylamide gel and analyzed by Western blotting using standard procedures. The primary antibodies used were: anti-DNMT1 (New England Biolabs), 1:2000; anti-DNMT3b (Abgent), 1:250; anti-actin (Abcam), 1:5000. Primary antibodies were visualized by ECL chemiluminescence (Perkin-Elmer) according to the manufacturer's protocol.

Example 9

NALM-6 and HCT116 cells were cultured under standard conditions in RPMI 1640 and McCoy's 5a medium, respectively. To analyze the effect of DNA methyltransferase inhibitors, cells were continuously cultivated in media supplemented with 10 μ M 5-azacytidine, RG108 or Δ C-RG108, as indicated. Cells were diluted 1:10 in fresh media every 3 or 4 days. For the determination of cellular growth and viability, cells were stained with trypan blue and counted using a standard counting grid.

Example 10

RG108 causes demethylation in HCT116 cells. We have used methylation-specific PCR (Herman, J.G., Graff, J.R., Myohanen, S., Nelkin, B.D., Baylin, S.B. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc. Natl. Acad. Sci. USA. 1996, 93:9821-9826) to determine the effect of RG108 on the methylation of the human hMLH1 gene in HCT116 cells. This revealed a complete demethylation of hMLH1 in cells treated with 10 micromolar RG108, while no demethylation was observed in control experiments without inhibitor or with 10 micromolar 5-azacytidine (Sigma), respectively (Fig. 5).

Example 11

RG108 reduces the proliferation of HCT116 cells (Fig. 6a), which can be partially attributed to the reactivation of the *p16^{ink4a}* tumor suppressor gene (Fig. 6b). In addition, RG108 substantially demethylates and reactivates euchromatic genes (e.g. tumor suppressor genes, Fig. 6c), but not the centromeric satellite sequences (Fig. 6d). This might be important for the maintenance of genome stability in cells/patients treated with RG108, because demethylation of satellite sequences has been shown to promote tumorigenesis by destabilizing chromosome organization (Ehrlich, M. DNA methylation in cancer: too much, but also too little. *Oncogene* 21, 5400-5413. (2002)).

The methylation status of satellite sequences was analyzed by methylation-sensitive Southern blots, as described previously (Rhee, I. et al. DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. *Nature*, vol. 416, pp. 552-556 (2002)).

Methylation-specific PCR analysis was performed as described previously (Myohanen, S.K., Baylin, S.B. & Herman, J.G. Hypermethylation can selectively silence individual p16^{ink4A} alleles in neoplasia. *Cancer Res*, vol. 58, pp. 591-593 (1998). Suzuki, H. et al. Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. *Nat Genet*, vol. 36, pp. 417-422 (2004). Bachman, K.E. et al. Methylation-associated silencing of the tissue inhibitor of metalloproteinase-3 gene suggest a suppressor role in kidney, brain, and other human cancers. *Cancer Res*, vol. 59, pp. 798-802 (1999)).

Total RNA was isolated from cells using Trizol reagent (Invitrogen). cDNA was synthesized by using the cDNA synthesis kit from Invitrogen. RT-PCR was performed as described previously (Rhee, I. et al. DNMT1 and DNMT3b cooperate to silence genes in

human cancer cells. *Nature*, vol. 416, pp. 552-556 (2002). Suzuki, H. et al. Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. *Nat Genet*, vol. 36, pp. 417-422 (2004)).

5 **Example 12**

Bisulfite sequencing was performed under standard conditions (Frommer, M., et al., 1992. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc. Natl. Acad. Sci. USA*, 89: 1827-1831) with genomic DNA from HCT116 cells treated with 30 μ M RG108 or no inhibitor for 5 days. The
10 primers used and the PCR conditions were as follows: forward TTTGTTTTTTTAGTTTTTGT, reverse AATCCCCCAAACCTCCAAC, 95 °C 3 min., 38 cycles (95 °C 30 s, 58 °C 30 s, 72 °C 30 s), 72 °C 5 min. PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen), subcloned into the pCR 4-Topo plasmid vector (Invitrogen) and subjected to automated sequencing. The results are shown
15 in Fig. 7. The analysis revealed significant demethylation of the CpG dinucleotides of the TIMP-3 CpG island in RG108 treated cells ($P < 0.05$, as determined by a t-test).